1982

Use of Ion-pairing for High Performance Liquid Chromatography of Alkaloids and Quarternary Ammonium Compounds

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USE OF ION-PAIRING FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF ALKALOIDS AND QUATERNARY AMMONIUM COMPOUNDS

BY

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A thesis submitted
in partial fulfilment of the requirements for the
degree Master of Science
Major in Chemistry
South Dakota State University
1982

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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USE OF ION-PAIRING FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF ALKALOIDS AND QUATERNARY AMMONIUM COMPOUNDS

INTRODUCTION

The alkaloids have been used by mankind in potions, medicines, teas and poisions for at least 3000 years. They occur throughout the plant and animal kingdoms and display a diversity of structure unmatched by any other group of natural compounds. Because the alkaloids exhibit an extraordinary array of pharmacological activities they are widely used in medicine. The following list is designed to indicate the degree of use of various alkaloids by U.S. physicians:

Major: Atropine, Ergonovine, Morphine, Codeine

Medium: Scopolamine, Ergotamine, Quinine, Cocaine

Minor: Strychnine, Brucine (Ref. 1)

A general approach to the ion-pairing high performance liquid chromatography (HPLC) of alkaloids and quaternary ammonium compounds is described in the analysis of these ionized solutes.

Over the years, a variety of different approaches have been developed to allow selectivity in liquid chromatographic separations, whether based on permeation, ion-exchange, adsorption or partitioning phenomena. The latter approach has gained wide usage recently due to the development of stable normal or reversed-phase systems. The application of reversed-phase systems certainly has become the most popular form of HPLC.

HPLC has been used previously for analysis of alkaloids using

normal-phase adsorption method. This often results in high solute retention coupled with very poor peak shape and solute resolution.

One of the major advantages of liquid chromatography over gas chromatography arises from the participation of the mobile phase in the equilibria distribution of the sample eluate molecules. Besides this primary chemical equilibria distribution process between mobile and stationary phase, secondary chemical equilibria between the eluate molecules and components present in either the eluent or the stationary phase can be established conveniently by choice of chromatographic conditions. These secondary processes will influence a variety of kinetic and thermodynamic parameters including ionization state, reversible complex formation, etc. Consequently, these secondary equilibria can have dramatic effects on the retention characteristics of the compound.

The desired qualities of the ion-pairing reversed-phase HPLC method for the analysis of ionized solutes are rapidity, sensitivity, efficiency and ability to resolve materials from complex systems without a prior solvent extraction step.

PURPOSE

The purpose of this investigation was to study the retention, peak symmetry and column efficiency of protonated alkaloids and quaternary ammonium compounds in a reversed-phase system. The mobile phase consisted of anionic species as counterions in a mixture of triethylamine buffer with an organic modifier. The retention was regulated by changing the counterion concentration and the pH of the mobile phase, as well as peak symmetry and column efficiency.

THEORETICAL CONSIDERATION

A. Ion-pair Formation

In any given liquid phase oppositely charged ions have some tendency to attract one another, depending upon the dielectric constant of the medium, the solvation of the individual ions and the types of interaction that cause ion association. Oppositely charged ions can be sufficiently bound to one another so that the resulting entity acts as a partially or fully neutralized species. Thus

$$Q^+ + C^- \rightleftarrows [QC] \qquad (1)$$

where [QC] represents the ion-pair species.

The extraction of alkaloids as ion-pairs was first reported as early as 1931. The basic theory of ion-pair techniques has been discussed by Schill (Ref. 2). Although the theory for batch extraction of ion-pairs has been thoroughly confirmed, the exact mechanism for ion-pairing chromatography has not been clearly established to date. Two fundamental models have been proposed. The first postulates that the solute molecule forms an ion-pair with the counter ion in the mobile phase. This uncharged ion-pair then partitions into the stationary phase (Ref. 3). The other mechanism postulates that the counterion partitions into the stationary phase with its ionic group oriented at the surface. The column behaves like a liquid ion-exchange column, referred to as a dynamic ion-exchanger. In actuality, the true mechanism surely involves both postulates, but is probably made complex by adsorption micelle formation, and complexation of both the solute and the ion-pair reagent. In any case, for the practicing chromatographer, the formation

of the ion-pair in the mobile phase is conceptually the easiest model to understand. We will use this approach to develop the basic equations of ion-pair reversed-phase high performance liquid chromatography (IP-RPHPLC) for a cationic species.

A cation (Q^+) may be partitioned into a nonpolar stationary phase as an ion-pair (QC) with a counterion (C^-),

$$Q^+_{aq} + C^-_{aq} \rightleftarrows [QC]_{aq} \rightleftarrows [QC]_{org} \quad (2)$$

$$E_{QC} = [QC]_{org}/([Q^+]_{aq} \cdot [C^-]_{aq}) \quad (3)$$

where E_{QC} is the overall equilibrium constant. We can use the distribution ratio (D) to express solute in the two phases.

$$D = [QC]_{org}/[Q^+]_{aq} = E_{QC}[C^-]_{aq} \quad (4)$$

The retention time (Tr) of a solute in HPLC is given by:

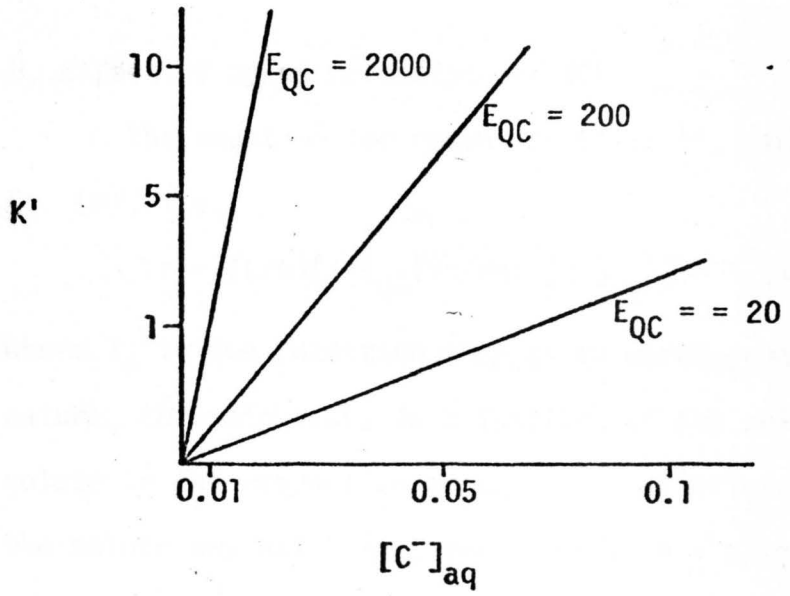
$$Tr = (L/v)(1+K') \quad (5)$$

where L is the length of the column, v is the velocity of the eluent, and K' is the capacity ratio, defined as the number of moles of solute in the stationary phase divided by the moles in the mobile phase,

$$K' = D(Vs/Vm) = E_{QC}[C^-]_{aq}(Vs/Vm) \quad (6)$$

where Vs and Vm are the volumes of stationary and mobile phase. Eq.(6) is plotted in Fig. 1, as a function of various concentrations of $[C^-]_{aq}$, assuming no competing reaction occurs (Ref. 4). As shown in Fig. 1, the K' values of the ion-pairs having low E_{QC} values cannot be easily altered by changing counterion concentration; for high E_{QC} values separation can be easily made at low concentration. Substituting Eq. (5) into Eq.(6).

Fig. 1 Regulation of the retention of a cation by the extraction constant and the concentration of the counterion in reversed-phase IP-HPLC.



$$Tr = (L/v)[1+(Vs/Vm)(E_{QC}\cdot[C^-]_{aq})] \qquad (7)$$

From Eq. (7) it may be seen that retention may be controlled by the stability of the ion-pair E_{QC} and the concentration of the counterion. As the concentration of the counterion is increased the retention time should also increase, but experimental results reported here and elsewhere (Ref. 5) show that for certain counterions above a maximum concentration Tr may actually decrease. The predicted linear dependance

of the capacity ratio and retention time on the concentration of the counterion was not strictly observed and secondary chemical equilibria (SCE) have been invoked to explain the results. The SCE add a new dimension to modern liquid chromatography, and much work is underway to incorporate past advances along with new approaches into HPLC models.

B. Effect of pH on Retention and SCE

The equation for retention time, Tr, can be written in the form Eq. (8), i.e.,

$$Tr = (L/v)[1+E_{QC}(Vs/Vm)([C^-]_{aq})] = T_o(1+K') \qquad (8)$$

where T_o is the retention time of an unretained component. By its very nature, chromatography is a function of the relative number of moles of solute in the respective phases at equilibria. When SCE are involved, the solute may exist in several forms in a given phase. In such a case, the distribution ratio(D),i.e.,

$$D = \frac{\text{amount of solute in stationary phase}}{\text{amount of solute in mobile phase}} \qquad (9)$$

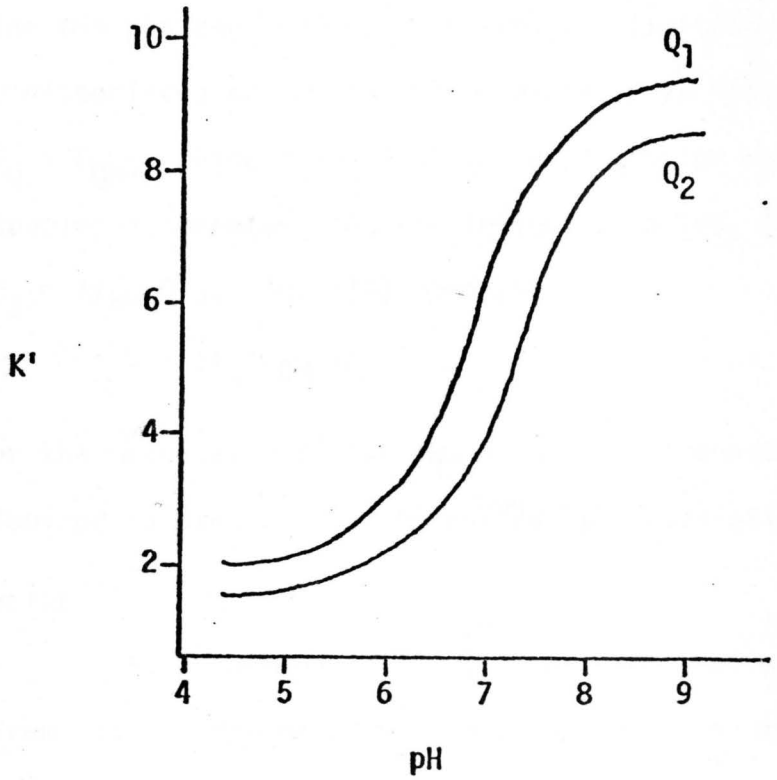
should be substituted for E_{QC} in Eq. (3).

In order to illustrate how D is employed, consider the distribution of a weak base Q between an aqueous mobile phase and an organic stationary phase via ionization control. Three equilibria can be assumed to be involved:

$$H_3O^+ + Q_{aq} \rightleftarrows H_2O + QH^+_{aq} \qquad E_I \qquad (10)$$

$$Q_{aq} \rightleftarrows Q_{org} \qquad E_Q \qquad (11)$$

Fig. 2 Retention and selectivity of two bases in the reversed-phase model as a function of pH.



$$QH^+_{aq} \rightleftarrows QH^+_{org} \qquad E_{QH^+} \qquad (12)$$

The extraction of QH^+ into organic phase may be due to ion-pair formation, but we shall not consider this point here. The distribution then can be written as:

$$D = \frac{[Q]_{org} + [QH^+]_{org}}{[Q]_{aq} + [QH^+]_{aq}} \qquad (13)$$

substituting the mass action expression for E_I, E_Q, E_{QH^+} leads to,

$$D = \frac{E_Q}{1+E_I \cdot [H_3O^+]} + \frac{E_{QH^+} \cdot [QH^+]_{aq}}{[Q]_{aq}(1+E_I \cdot [H_3O^+])} \qquad (14)$$

thus substituting Eq. (13) into Eq. (7), leads to the appropriate retention time dependance. A plot of K' vs pH for two bases, which can be directly obtained from Eq. (6) and Eq. (14), is shown in Fig. 2. The two plateau regions represent the limiting capacity factors of the undissociated and dissociated species. In Fig. 2, we assume that $E_Q > E_{QH^+}$, which means that the distribution ratio of the undissociated species is greater. At the inflection point, $[QH^+]_{aq} = [Q]_{aq}$ and $E_I = 1/[H_3O^+]$. Eq. (14) becomes:

$$D = (E_Q + E_{QH^+})/2 \qquad (15)$$

or the mean value of the two distribution constants. Retention of ionized solutes can be controlled by manipulation of pH near the $\log E_I$ value.

At intermediate pH, a large degree of separation can be obtained. From Fig. 2, the maximum separation arises from Q_2 existing to a large extent in the unionized form, whereas Q_1 is predominantly ionized. Here we take advantage of differences in E_I values for the achievement of separation. Thus in this example by simple control of pH values there are many possibilities for resolution of the two bases.

Many ion-pair separations show pronounced pH dependency. This is not surprising for solutes which can undergo proton ionization equilibria. In ion-pairing chromatography the conterions are usually strong electrolytes while the sample solutes are often weak acids or bases, therefore the pH of the mobile phase influences the retention. Our hypothetical weak base Q (in these experiments as alkaloids, usually in the form of tertiary amines) may be protonated to give a

quaternary ammonium species QH^+,

$$Q + H^+ \quad QH^+ \qquad (16)$$

then the effective concentration distribution of ion-pair ($QH^+ \cdot C^-$) between the phases will be dependent on the pH value.

C. Effect of the Composition and Concentration of Counterion on K'

The theoretical models for reversed-phase systems predict that K' can be varied over a wide range by the choice and concentration of the counterion. Ideally, the counterion should be univalent and soluble in the organic phase, and should not undergo aggregate formation or other secondary chemical equilibria (Ref. 6). Also it should be chemically compatible with the mobile and stationary phases and should not interfere with the detection systems. As far as detection is concerned, the formation of ion-pairs with the counterion of high molar absorptivity can increase detection response.

The ion-pair chromatographic separation of alkaloids and quaternary ammonium compounds can be achieved by a wide range of anionic counterions. For hydrophobic amine compounds, small hydrophilic counterions have been used successfully. The tendency toward ion-pairing formation for inorganic counterions is $Cl^- < Br^- < I^- < ClO_4^-$. Because small inorganic anions give low extraction constant values with hydrophobic cations, these anions are very useful for normal-phase separation (Ref. 6). Perchlorate, sulfate and phosphate are suitable for ion-pair reversed-phase separation because of their higher extraction constant. The long chain alkylsulfates and sulfonates have detergent properties; their use in

ion-pairing chromatography has been referred to as "soap" or "detergent" chromatography. They generally give higher E_{QC} values than do other anions of comparable alkyl chain length.

Eq. (6) predicts that K' is proportional to the counterion concentration in reversed-phase systems. Qualitative agreement with these predictions have been observed with bulk reversed-phase separations of basic and acidic solutes. Furthermore, the retention of a solute in reversed-phase systems will increase as the extraction constant E_{QC} increases. Thus to obtain a K' between 5 and 50 at a counterion concentration between 0.01 and 0.1M, an E_{QC} values of 50~5000 will be required (Ref. 10). Nonlinear plots of K' versus $[C^-]$ have been observed with ion-pair separation under partition conditions and the divergence from linearity has been attributed to secondary chemical equilibria in order to explain the results.

D. Peak Shape and Secondary Chemical Equilibria

By far the most significant problem with ion-pairing HPLC is the asymmetry or tailing of sample peaks. This is generally attributable to competing secondary equilibria such as dissociation in the organic phase, dimerization of pairing ions, slow kinetics in the chemical equilibria steps, etc.

OPERATION OF ION-PAIRING REVERSED-PHASE COLUMN

Columns recommended for ion-pairing reversed-phase are monomeric C_{18} or C_8 phases. The recommended mobile phase is buffered aqueous counterion solution with methanol used in the pH range of 2∿7.2, although acetonitrile can also be used as an organic modifier. Perchlorate, sulfate and phosphate were used for ion-pairing cationic species. However, dodecyl sulfate may give better efficiency, when acetonitrile is used as an organic modifier.

There are many other factors which enter into an ion-pairing reversed-phase HPLC separation which have been briefly described as shown in Table I (Ref. 3).

The control of the pH, as well as the concentration of counterion, is a most important parameter, since it will dictate the concentration of the ionic form of the solute. Obviously, for greatest retention we desire maximum ionization. For quaternary ammonium ions (pKa<8) which are ionized throughout the entire pH range so that the actual pH selected is dependent on other types of solutes present.

Mobile phases were buffer solutions of perchlorate-, sulfate-, or phosphate- with triethylamine in methanol or acetontrile (50:50). Triethylamine was used as a base to adjust to the desired pH values. The solutions were filtered through a millipore filter to remove any undissolved particles or impurities and finally degassed.

Table I. Predicted Variables of IP-RPHPLC Separation

Variable	Effect
Type of counterion	The better the ability to ion-pair, the longer the retention.
Size of counterion	An increase in the size of counterion will increase retention.
Concentration of counterion	Increasing concentration increases retention up to a limit.
pH	Effect is dependent on nature of solute; retention increases as pH maximizes concentration of ionic form of solute.
Type of organic modifier	Retention increases with increasing viscosity.
Concentration of organic modifier	Retention decreases with increasing concentration of organic modifier.
Stationary phase	The more lipophilic or less polar the stationary phase, the longer a nonpolar compound is retained.

Table II. Compounds Used as Samples in Experiments.

Type	Compound	Systematic Name	pKa	Ref.
Tertiary amine	Strychnine	$C_{21}H_{22}N_2O_2$	6.0	Merck Index
Tertiary amine	Brucine	2,3-dimethoxy strychnidin -10-one, $O_{23}G_{26}N_2O_4$	6.04	"
Tertiary amine	Nicotine	(S)-3-(1-methyl-2-pyrrolidinyl pyridine, $C_{10}H_{14}N_2$	6.16	"
Tertiary amine	Atropine	endo-(t)-2-(hydroxymethyl) benzene acetic acid 8-methyl-8-azabicyclo[3,2,1] oct-3-yl -ester, $C_{17}H_{23}NO_3$	4.35	"
Tertiary amine	Ergonovine	9.10-Didehydro-N-(2-hydroxy-1-methyl)-6-methyl ergoline-8-3-(S) -carboxamide, $C_{19}H_{23}N_3O_2$	6.8	"
Tertiary amine	Ergotamine	12'-Hydroxy-2'-methyl-5' α-(phenyl methyl)ergotamine -3',6',18 trione, $C_{33}H_{35}N_5O_5$	--	"
Tertiary amine	Ergocryptine	$C_{32}H_{41}N_5O_5$	--	"
Quaternary amine	Ergothioneine	$C_9H_{15}N_3O_2S$	--	"
Quaternary amine	Paraquat	1,1'-dimethyl-4,4'-bipyridium dichloride $C_{12}H_{14}Cl_2N_2$	--	Farm Chemical Handbook

Table II. (Cont.)

Type	Compound	Systematic Name	pKa	Ref.
Quaternary amine	Difenzoquat	1,2-methyl-3,5-diphenyl -1H-pyrozolium methyl sulfate, $C_{18}H_{20}N_2O_4S$	--	Farm Chemical Handbook
Quaternary amine	Diquat	6,7-dihydrodipyridol [1,2-a,2',1'-c] pyrazidinum dibromide monohydrate, $C_{12}H_{12}Br_2 \cdot H_2O$	--	"

EXPERIMENTAL

Apparatus:

A Model M-600 (Water Association, Milford, MA) HPLC was used with a Model 6000A dual plunger pump and a Model U6K universal liquid chromatography injector.

A Model 153 Analytic UV (Beckman, Fullerton, CA) fixed wavelength detector at 254 nm and Beckman Model 1005 recorder were used.

A C_{18} (Alltech Associates, Deerfield, IL) column packed with 10μ Alltech packing material was used as an alkyl-bonded reversed-phase column. The column was 25cm long and had a 1/4 in O.D. and 4.6mm I.D.

The pH measurements were performed with a Corning Model 12 Research pH meter.

Chemicals and Reagents:

All reagents and solvents were analytical-reagent grade and used without further purification, except triethylamine which was redistilled before use. The compounds used as samples are listed in Table II.

Equilibration:

In these experiments the concentration of ion-pairing reagent was maintained between 0.1∿0.01M. The type of counterion and organic modifier were studied. In all cases a minimum of 20∿30ml of mobile phase was pumped through the column before making injection.

RESULTS AND DISCUSSION

The alkaloids may be protonated to form quaternary ammonium ions. The separation of these protonated alkaloids, and also quaternary ammonium compounds by means of reversed-phase chromatography is difficult because of the strong interactions of these catonic species with the adsorption sites of the stationary phase. When pure methanol was used as the mobile phase, the quaternary amines could not be eluted at all, while the protonated alkaloids showed broad tailing peaks with very long retention times. With suitable counterions such as perchlorate, sulfate and phosphate, which are completely ionized in the pH 2∿8 range, ion-pairs are formed. The electrically neutral compounds formed can be retained to a certain extent on a reversed-phase column.

The addition of a suitable counterion within the range of 0.1∿0.01M may provide a stable ion-pair formation constant, thus all samples could be eluted in sharp, relatively symmetrical peaks and in reasonable time. Figs. 3A and 3B show the separation of selected alkaloids on C_{18} bonded reversed-phase column.

In Fig. 3A the separation of 2 pairs of closely related compounds, i.e., strychnine and brucine, atropine and scopolamine, shows good selectivity. As shown in Fig. 3B, use of acetonitrile as an organic modifier gave better efficiency and peak symmetry. Because acetonitrile has a remarkably low viscosity and is a good solvent, the ion-pairs eluted relatively fast and appeared as symmetrical peaks. Similar results can be obtained for the rest of the alkaloids, except for ergothioneine which is relatively high polar,so the retention time is very

short.

The quaternary ammonium compounds, such as paraquat, diquat and difenzoquat showed bad tailing when,acetonitrile was used as an organic modifier in the mobile phase, especially when pH was high.

The presence of a fused heterocyclic ring system in alkaloids

Fig. 3A IP-RPHPLC of alkaloids; Br = Brucine, St = Strychnine, Sc = Scopolamine, At = Atropine; Mobile phase: 0.05M perchlorate-triethylamine buffer/methanol (50:50); pH 5.5; Flow rate 1ml/min.. The amount of sample injected is between 1μℓ and 10μℓ at concentrations of 1mg/ml.

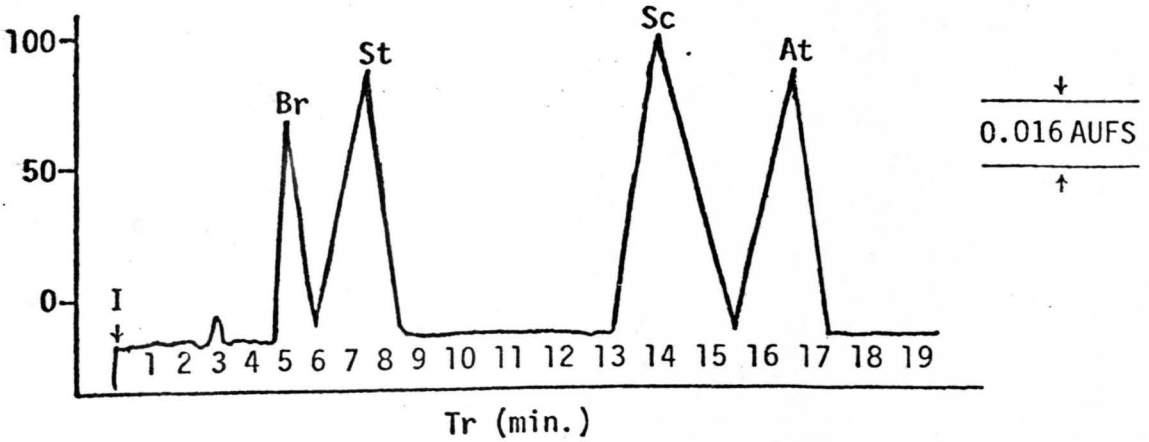


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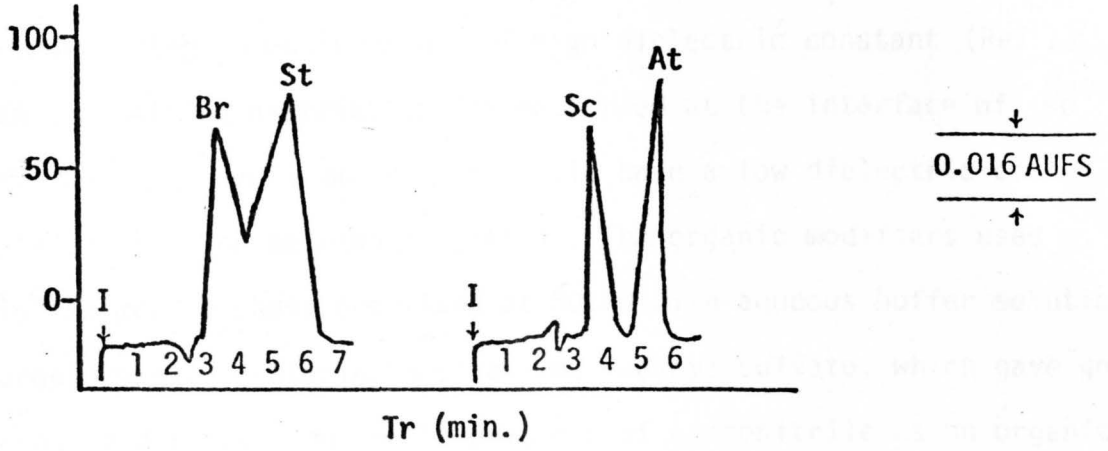
Fig. 3B IP-RPHPLC of alkaloids; Br = Brucine, St = Strychnine, Sc = Scopolamine, At = Atropine; Mobile phase: 0.05M perchlorate-triethylamine buffer/acetonitrile (50:50); pH 5.5; Flow rate 1ml/min.



had a major influence on retention and the order of elution was strongly influenced by the nature of the nitrogen atom, i.e., the major discriminating factor is the number and the basicity of nitrogen atoms. Relatively small hydrophilic inorganic anions such as perchlorate, sulfate and phosphate,may form stable ion-pairs with a large variety of cationic quaternary ammonium ions, but their extremely high hydration will prohibit ion-pairing in environments of high dielectric constant (Ref.7) . Such an ion-pairing association may be formed at the interface of two phases. Thus the mobile phase used should have a low dielectric constant to promote ion-pairing formation. The organic modifiers used here in the mobile phase are fixed at 50:50 with aqueous buffer solutions. One large organic counterion was tested, dodecyl sulfate, which gave good efficiency and resolution in the presence of acetonitrile as an organic modifier as shown in Fig. 4A and Fig. 4B.

The lower solubility of sodium dodecyl sulfate in methanol may cause interference with the stationary phase. When methanol was employed as an organic modifier, strychnine, brucine and quaternary ammonium compounds did not elute at all. It is interesting to note that atropine and scopolamine have only subtle molecular structure differences. When large counterions are used, these differences may be masked, thus exhibiting the same retention behavior as shown in Fig. 4B.

The capacity ratio varied with the type of counterions, as can be seen in Table III. High K' values are considered to be an indication of stronger ion-pairs. These observations seem to indicate that perchlorate and sulfate ions can form stronger ion-pairs with most of the compounds

Fig. 4A IP-RPHPLC; Sc = Scopolamine, At = Atropine, Br = Brucine, St = Strychnine; Mobile phase: 0.01M Sodium Dodecyl Sulfate-triethylamine/acetonitrile (50:50); pH 3.5; Flow rate 1ml/min.

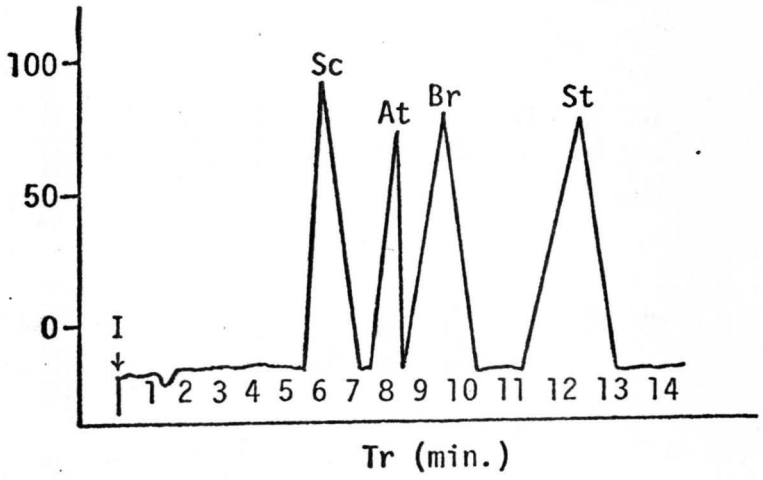


Fig. 4B Methanol used as organic modifier.

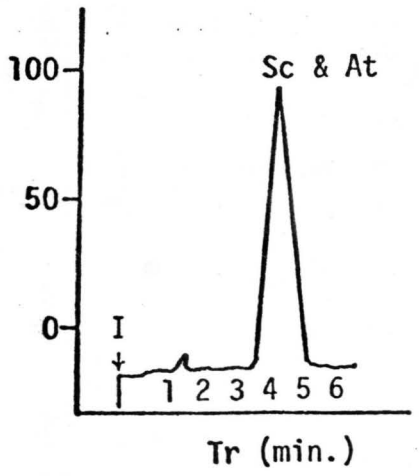


Table III. Capacity ratio of samples in three different counterion systems.

Counterion	Conc.	pH	Strychnine	Brucine	Nicotine	Paroquat	Scopolamine	Atropine
Perchlorate	0.01M	4.5	4.13	3.6	1.73	4.47	1.53	2.67
	0.05M	4.5	2.1	1.4	0.6	0.33	0.33	0.93
	0.1M	4.5	1.8	1.67	0.4	0.2	0.67	0.8
Sulfate	0.01M	4.5	4.3	3.8	1.4	2.67	0.93	1.67
	0.05M	4.5	2.1	1.7	0.46	0.8	0.06	0.43
	0.1M	4.5	2.3	2.0	0.6	0.73	0.6	0.87
Phosphate	0.01M	4.5	5.27	5.1	1.67	8.2	1.27	2.2
	0.05M	4.5	3.47	2.87	0.73	1.53	0.53	0.93
	0.1M	4.5	3.6	3.2	0.87	1.47	0.6	1.3

than phosphate ion. It is recommended that in the phosphate system the counterion concentration should be high (>0.05M) and the pH of the mobile phase should be low (<4.5). Then the formation of stable ion-pairs would be promoted, which provides reproducibility.

In order to determine the nature of the chromatographic mechanism, the effects of the counterion in the mobile phase on the capacity ratio K' were investigated. We consider the model of ion-pairing formation in the mobile phase,M followed by the partition of this ion-pair on the stationary phase,S. Thus assuming again that Q^+ is the solute ion and C^- is the counterion, we have,

$$Q_M^+ + C_M^- \rightleftarrows (Q^+C^+)_M \qquad K_{IP} \qquad (17)$$

$$(Q^+C^-)_M \rightleftarrows (Q^+C^-)_S \qquad K_{QC} \qquad (18)$$

$$Q_M^+ \rightleftarrows Q_S^+ \qquad K_{Q^+} \qquad (19)$$

again where K_{IP} is the ion-pairing constant, K_{QC} is the distribution constant of the ion-pair to the stationary phase, and K_{Q^+} represents the retention of the solute species with counterion present. From Eq. (9), the distribution ratio can then be written as:

$$D = \frac{[Q^+]_S+[Q^+C^-]_S}{[Q^+]_M+[Q^+C^-]_M} \qquad (20)$$

and substituting Eq. (17), (18), (19),(20), into Eq. 6, we have

$$K' = \left(\frac{VS}{VM}\right)\left(\frac{K_Q+K_{IP}K_{QC}[C^-]_M}{1+K_{IP}[C^-]_M}\right) \qquad (21)$$

From this equation it can be seen that in ion-pairing partition. K' has a rectangular hyperbolic relationship to concentration of the counterion. This was frequently observed throughout the experiments.

Thus the retention of quaternary ammonium ions can be regulated to a certain extent by the concentration of counterion in the mobile phase as demonstrated in Fig. 5. The curves show the relationship between K' and concentration of counterion. In these experiments 12 samples have been tested by 5 different counterions under 3 different concentrations plus 3 different pH values and 2 kinds of organic modifiers. All the results show similarity to Eq. (21). This may indicate that the mechanism of chromatographic behavior is ion-pair formation in the mobile phase followed by partition of this ion-pair on the stationary phase. However, the ion-pair model is not adequately demonstrated solely from chromatographic capacity ratio measurements.

It is interesting to note that if ion-pair formation does not occur in the mobile phase, then the combination of Eqs. (17), (18), and (19) would lead to the expression for K' (Ref. 8),

$$K' = \left(\frac{VS}{VM}\right)\left(K_Q + E_{QC}[C^-]_M\right) \qquad (22)$$

which is similar to Eq. (6), except for the addition of the retention of Q itself by the stationary phase. This equation predicts a linear behavior of K' on $[C^-]_M$, which is clearly not the case in the bonded reversed-phase column. In addition, the side reactions of ion-pairing formation should be taken into consideration to account for the nonlinearity of this model.

The practical utility of solvents used in the mobile phase is obviously dependent upon their availability and cost. Methanol and acetonitrile, being the most commonly used water-miscible organic solvents, were used in these experiments. The organic modifier plays

Fig. 5 ○:Strychnine, □ : Brucine, △ : Nicotine, ● : Paraquat

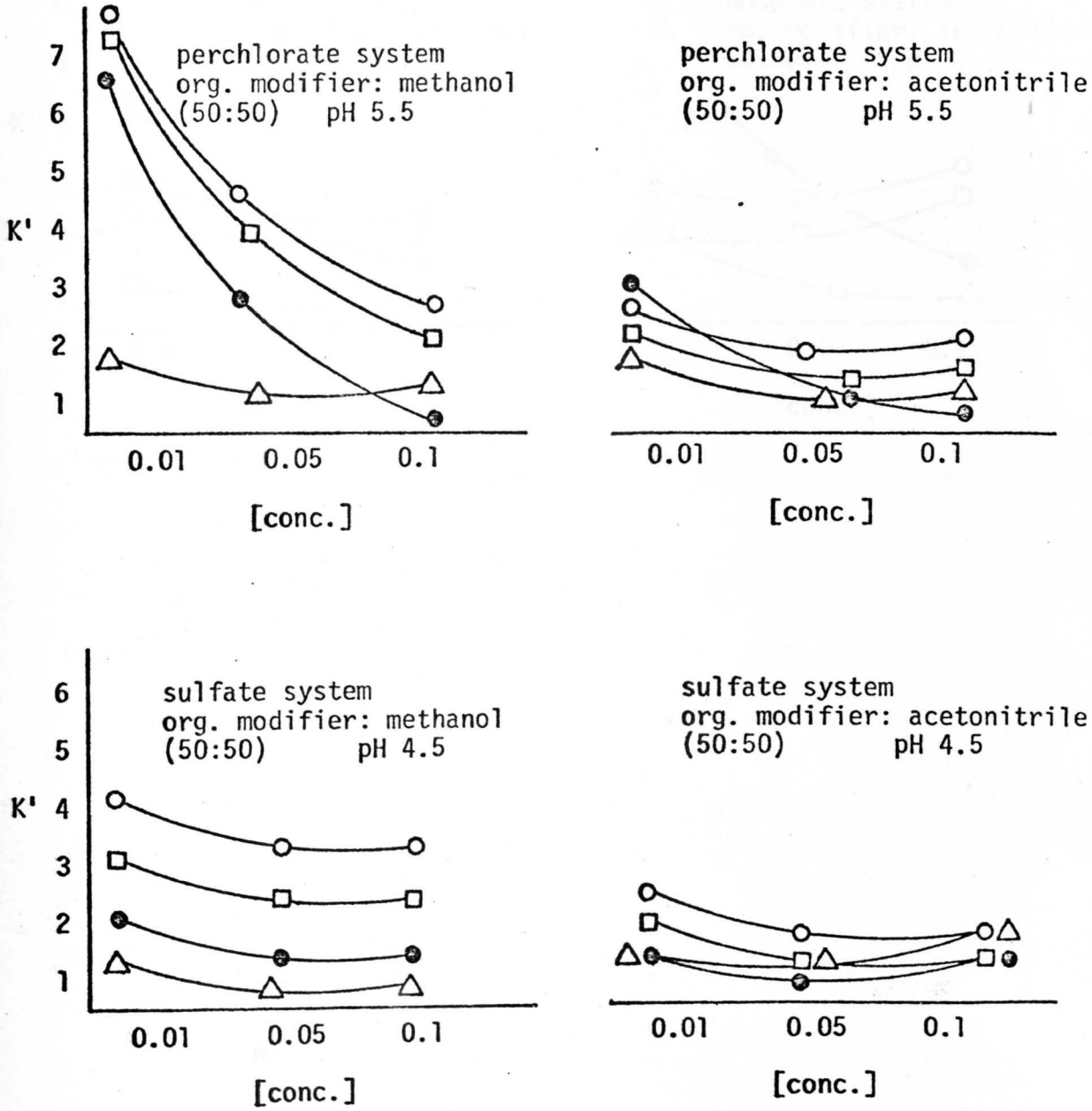
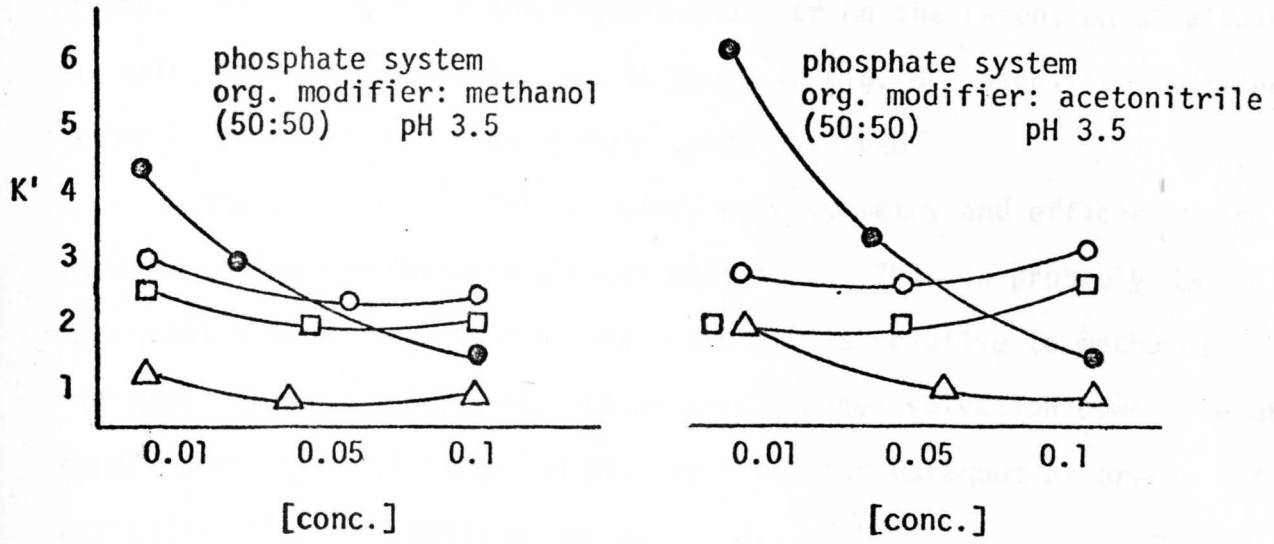


Fig. 5 (Cont.)


6
5
K' 4
3
2
1
phosphate system
org. modifier: methanol
(50:50) pH 3.5
0.01
0.05
0.1
[conc.]
phosphate system
org. modifier: acetonitrile
(50:50) pH 3.5
0.01
0.05
0.1
[conc.]

a significant role in determining chromatographic retention and selectivity. The influence of the organic modifier on the retention of alkaloids by using hydrophilic counterions is shown in Fig. 6. Significant differences in retention and peak symmetry were observed.

The changes in retention time, peak symmetry and efficiency are markedly different for both organic modifiers. This is probably due to the greater solvating power of the acetonitrile relative to methanol for quaternary ammonium ions. Given this greater solvation power, we might expect a better efficiency and peak symmetry for paraquat or brucine in acetonitrile than in methanol,as observed.

It is known that the organic modifier is extracted into the bonded phase (Ref. 9). It has been suggested that this extracted-imbedded layer provides a solvation medium for the extracted ion-pairs. As the organic solvent plays a significant role in ion-pairing extraction, we can ancitipate that the organic layer on the bonded phase will also be an important factor.

Chromatographic retention can be very sensitive to the pH of the mobile phase as shown in Fig. 2. When the species are partially ionized, small changes in pH markedly influence retention and selectivity. An important characteristic of ionization control via pH is the predictable manner of retention changes with variation of pH.

As an example, Fig. 7 shows the chromatographic retention of a series of substances as a function of pH. It is clear in this figure that there are a variety of mobile phase pH values that will lead to relatively easy separation.

Fig. 6 Retention and peak symmetry factor in different organic modifier as mobile phase. Flow rate, 1min/ml; sample concentration, 1mg/ml; injection amount 2~5μℓ.

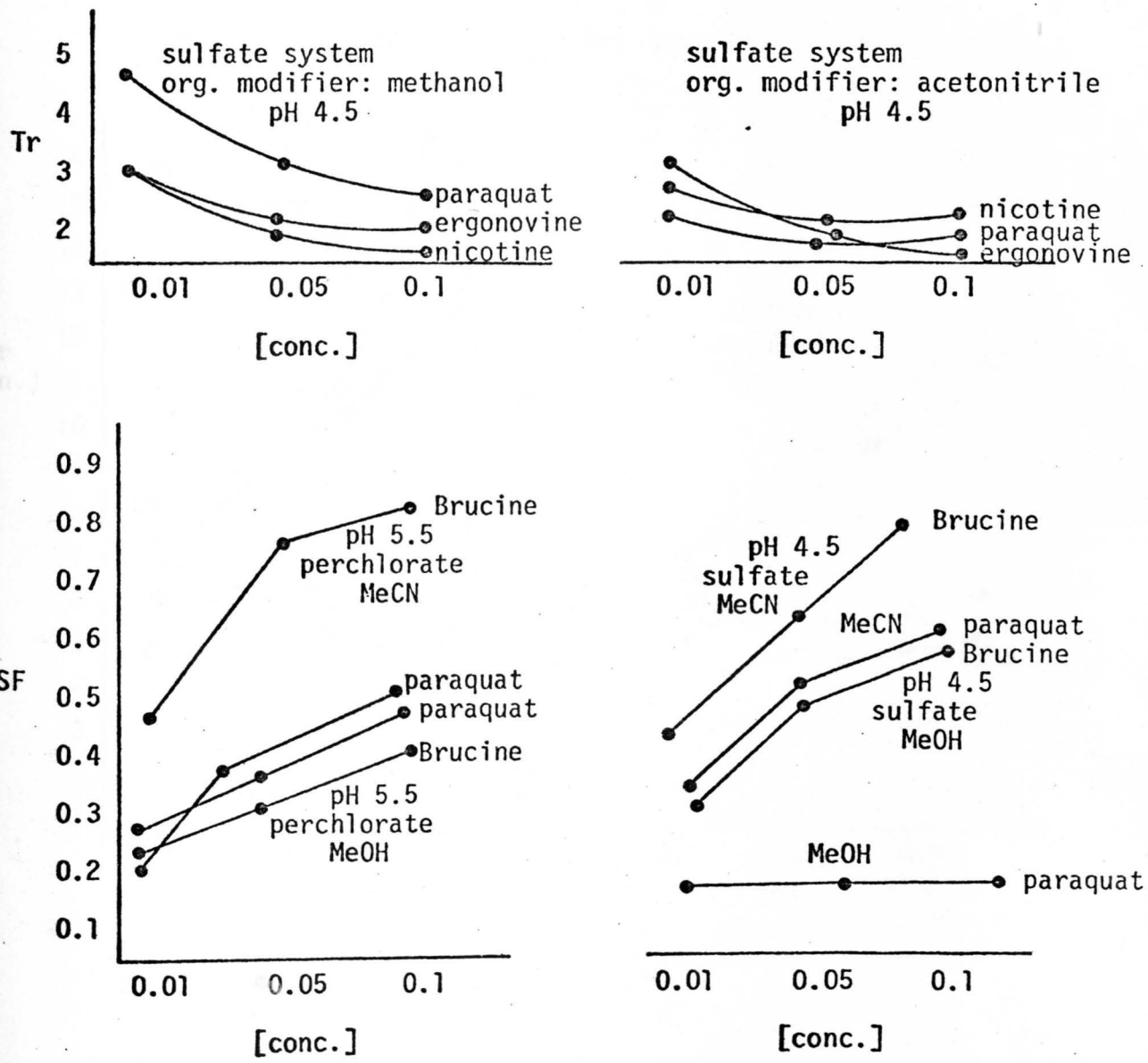


Fig. 7 Sample retention in three different mobile phases with methanol as a function of pH. A. 0.05M perchlorate, B. 0.05M sulfate, C. 0.05M phosphate system.

○ : strychnine □ : brucine

△ : scopolamine ● : atropine

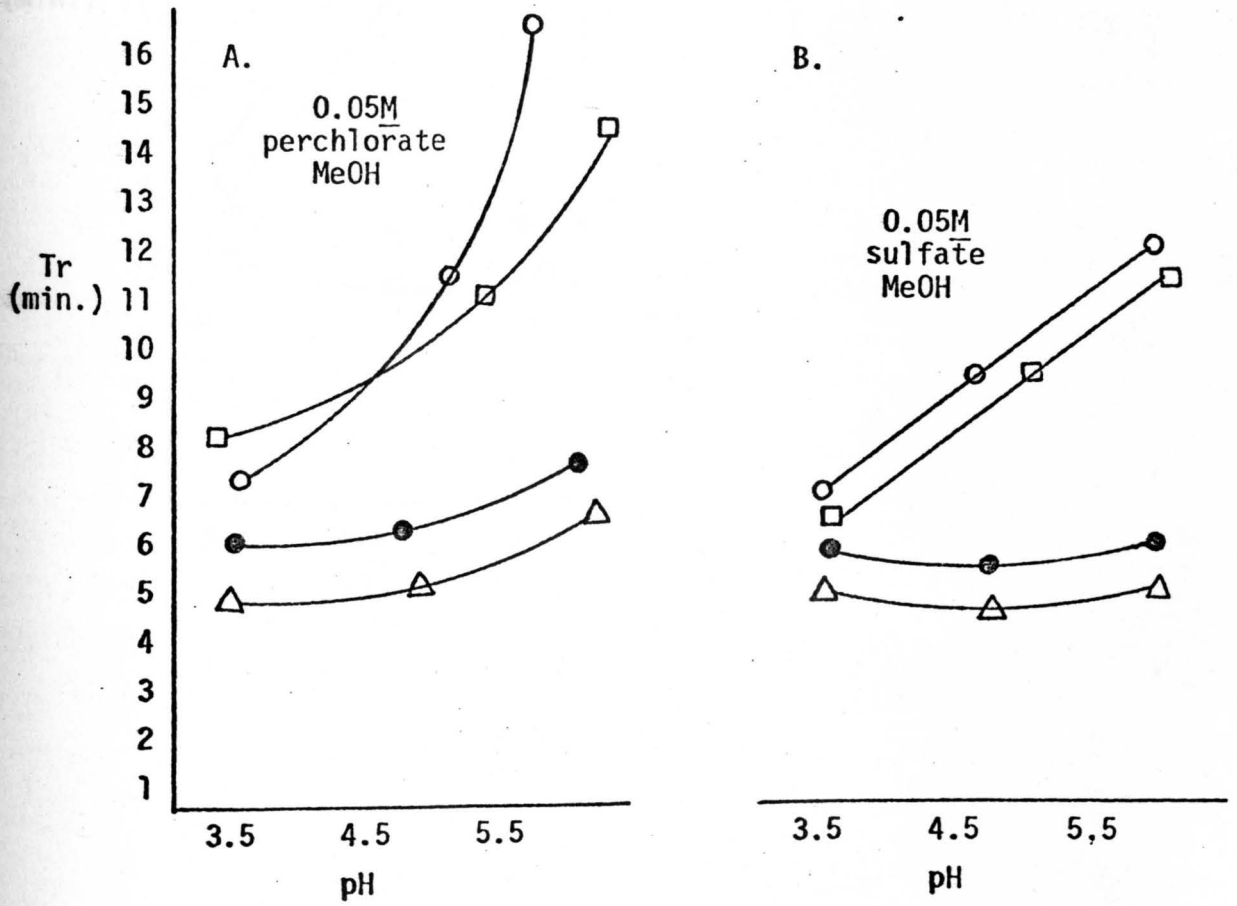
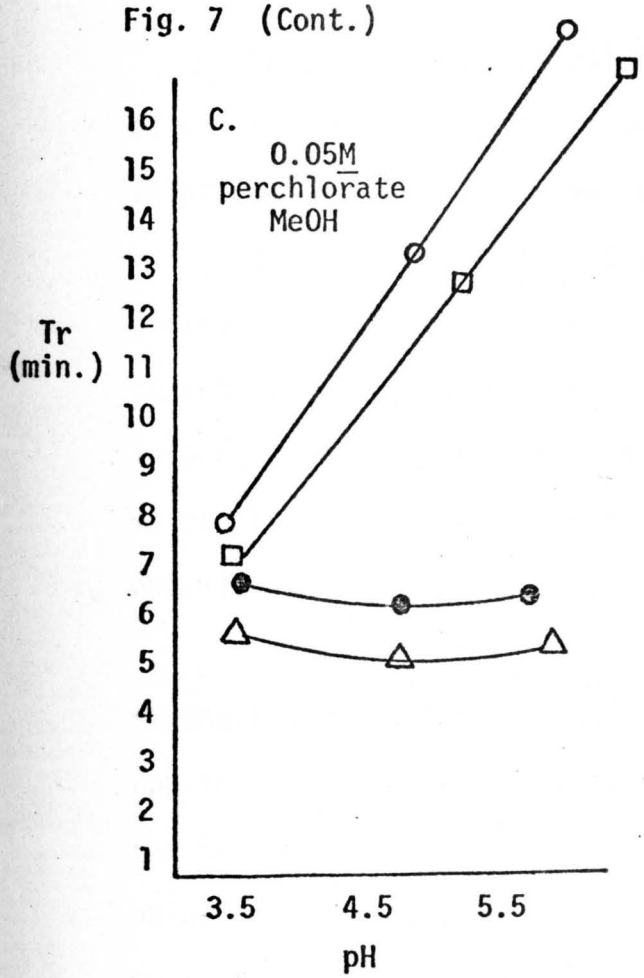


Fig. 7 (Cont.)


C.
0.05M
perchlorate
MeOH
Tr
(min.)
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
3.5
4.5
5.5
pH

A plot of the retention of strychnine and paraquat as a function of pH is shown in Fig. 8. As expected, the retention of paraquat remains relatively unchanged, whereas there is a significant change for strychnine. It appears from Fig. 8 that under chromatographic conditions the degree of proton ionization of the tertiary amine has a greater influence on retention than on quaternary ammonium compound. This may be considered in part from the difference in solvating of the respective charged groups. In practice, for maximum retention on a reversed-phase column one should operate at higher pH values.

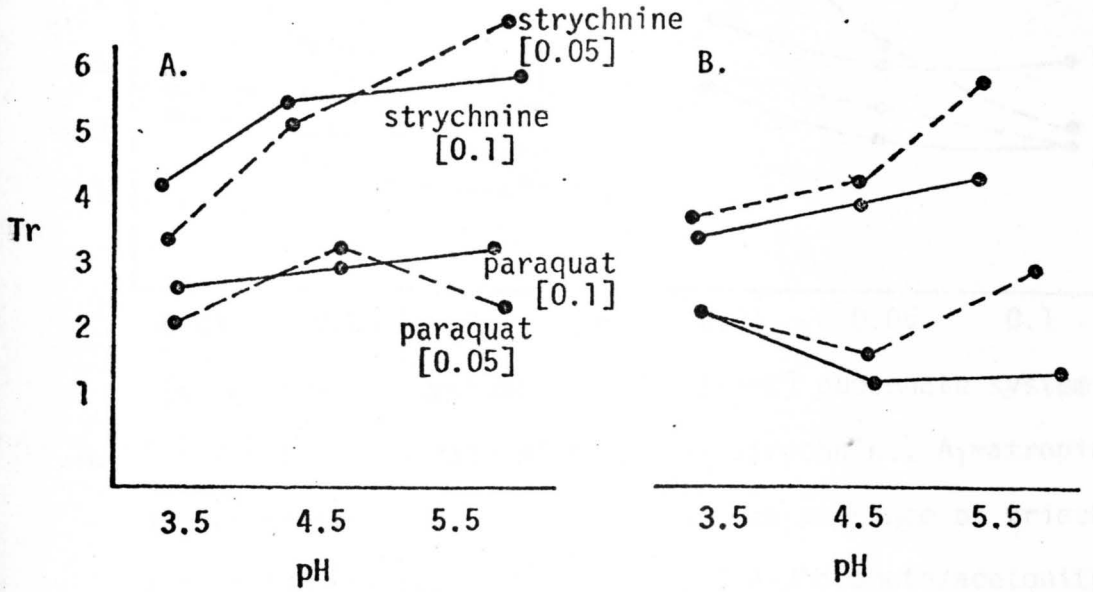
Retention reproducibility requires careful attention to the ionic strength and temperature which control pH and ionization constants, when applied to ion-pairing chromatography. The use of a buffer is essential for the accurate and precise control of pH. In these experiments triethylamine was used as a buffer and adjusted to the desired pH value. This not only yielded stable chromatographic systems but also enhanced retention reproducibility from mobile phase to mobile phase.

It is difficult to predict quantitatively the effect of ionic strength changes on the capacity ratio of an ion-pair complex. When ammonium sulfate is used as a counterion, a decrease in K' for an ion-paired solute would be expected for reversed-phase systems. As shown in Fig. 9 with increasing electrolyte concentration the possibility of appreciable ion-pairing formation between the solute and counterion also increases. Thus an increase in ionic strength decreases the transfer of ion-pairs between mobile phase and stationary phase.

Figure 10 shows peak symmetry of strychnine in the presence of

Fig. 8 A. Peak retention as a function of pH protonated tertiary amine, i.e., strychnine vs quaternary ammonium compound, i.e., paraquat in 0.1M, 0.05M sulfate system.

B. In 0.1M perchlorate system. Methanol used as organic modifier.



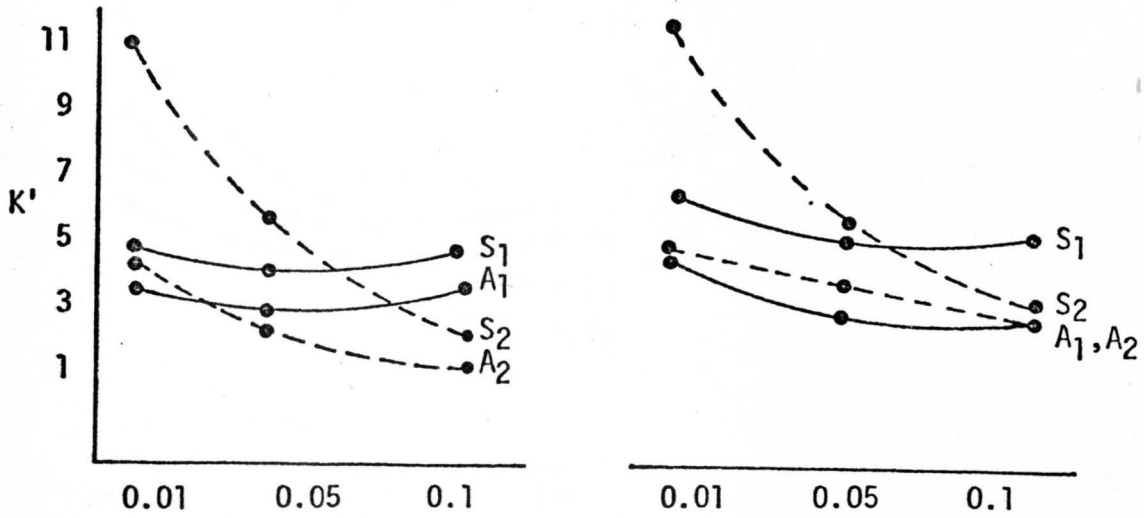
0.01M, sulfate system

—MeOH, ---MeCN

0.1M, perchlorate system

—MeOH, --- MeCN

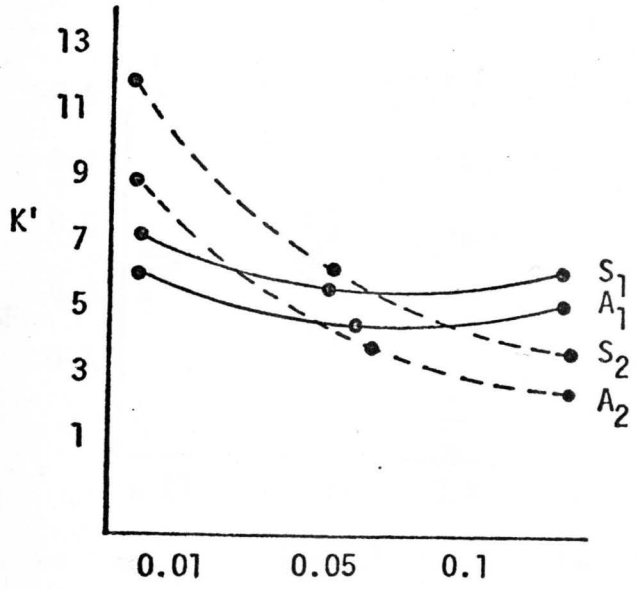
Fig. 9 Capacity ratio of samples as a function of counterion concentration.



A. S_1=strychnine, A_1=atropine in the presence of triethylamine-sulfate/acetonitrile system.
S_2=strychnine, A_2=atropine in the presence of ammonium sulfate as counterion.

B. S_1=strychnine, A_1=atropine in the presence of triethylamine-phosphate/acetonitrile system.
S_2=strychnine, A_2=atropine in the presence of ammonium sulfate as counterion.

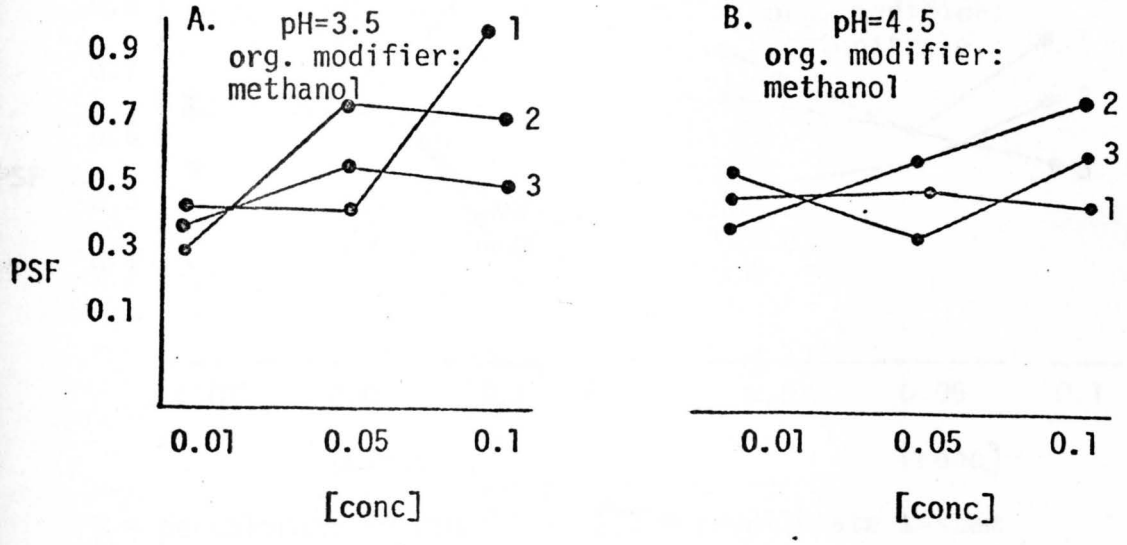
Fig. 9 (cont.)



[conc] perchlorate system

C. S_1=strychnine, A_1=atropine in the presence of triethylamine-perchlorate/acetonitrile system.
S_2=strychnine, A_2=atropine in the presence of ammonium sulfate.

Fig. 10 Peak symmetry factor of strychnine as a function of counter-ion concentration.



1 = perchlorate system

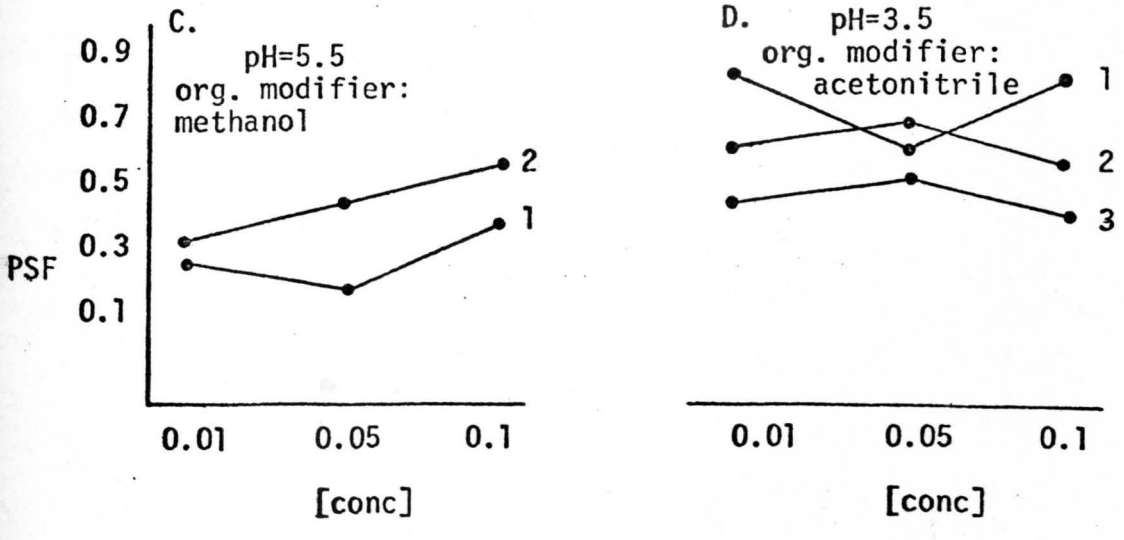
2 = sulfate system

3 = phosphate system

1 - perchlorate system

2 = sulfate system

3 = phosphate system



1 = perchlorate system

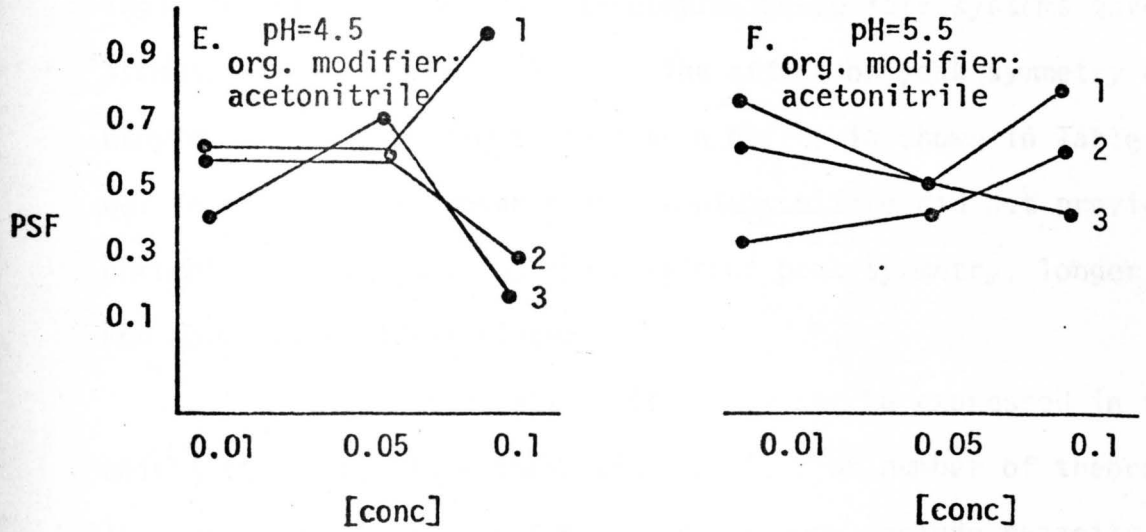
2 = sulfate system

1 = perchlorate system

2 = sulfate system

3 = phosphate system

Fig. 10 (cont.)



1 = perchlorate system

2 = sulfate system

3 = phosphate system

1 = perchlorate system

2 = sulfate system

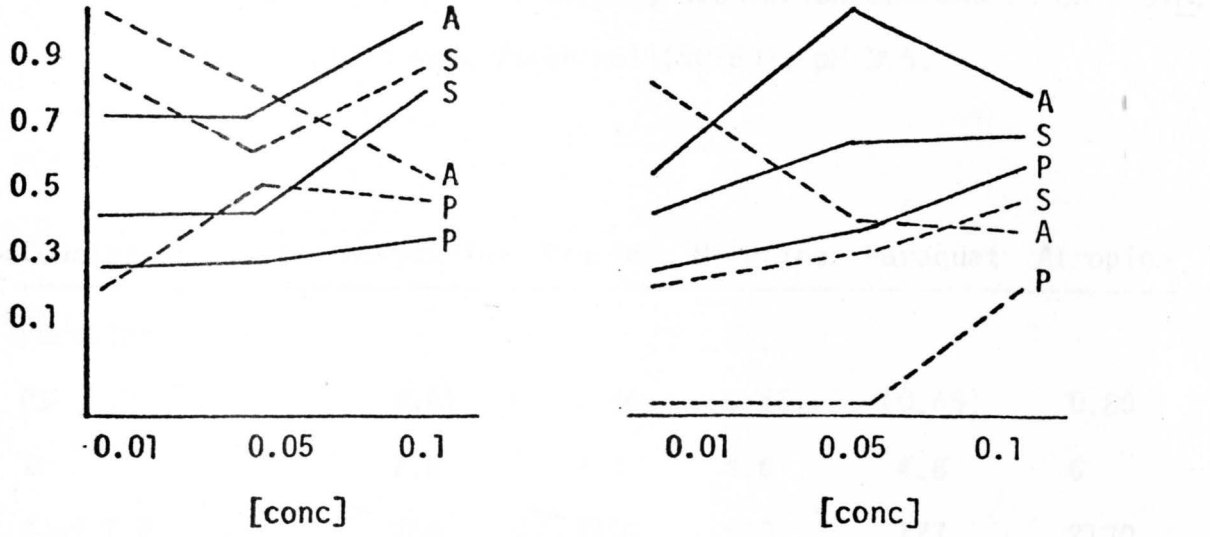
3 = phosphate system

different counterion systems. Use of acetonitrile as an organic modifier shows a better peak symmetry factor (length of peak leading vs peak trailing). Figure 11 shows that triethylamine-sulfate systems gave better peak symmetry than ammonium sulfate. The effect on peak symmetry of using triethylamine and ammonium sulfate as a buffer is shown in Table IV. It was found that the presence of ammonium sulfate did not provide a stable chromatographic system as shown by poor peak symmetry, longer retention and fewer theoretical plates.

The column separation efficiency can be expressed in terms of the height equivalent to a theoretical plate H or number of theoretical plates. The variables which affect column efficiency include capacity ratio, flowrate, pressure, temperature, sample elution time, mobile phase viscosity, etc. with ion-pair systems additional parameters include sample concentration, type of counterion and its concentration.

Figure 12 shows the column efficiency as related to both the nature of counterion and its concentration.

Fig. 11 Peak symmetry factor as a function of counterion concentration.
A=Atropine, S=Strychnine, P=Paraquat



A. —— methanol
---- acetonitrile
counterion: perchlorate
pH = 3.5

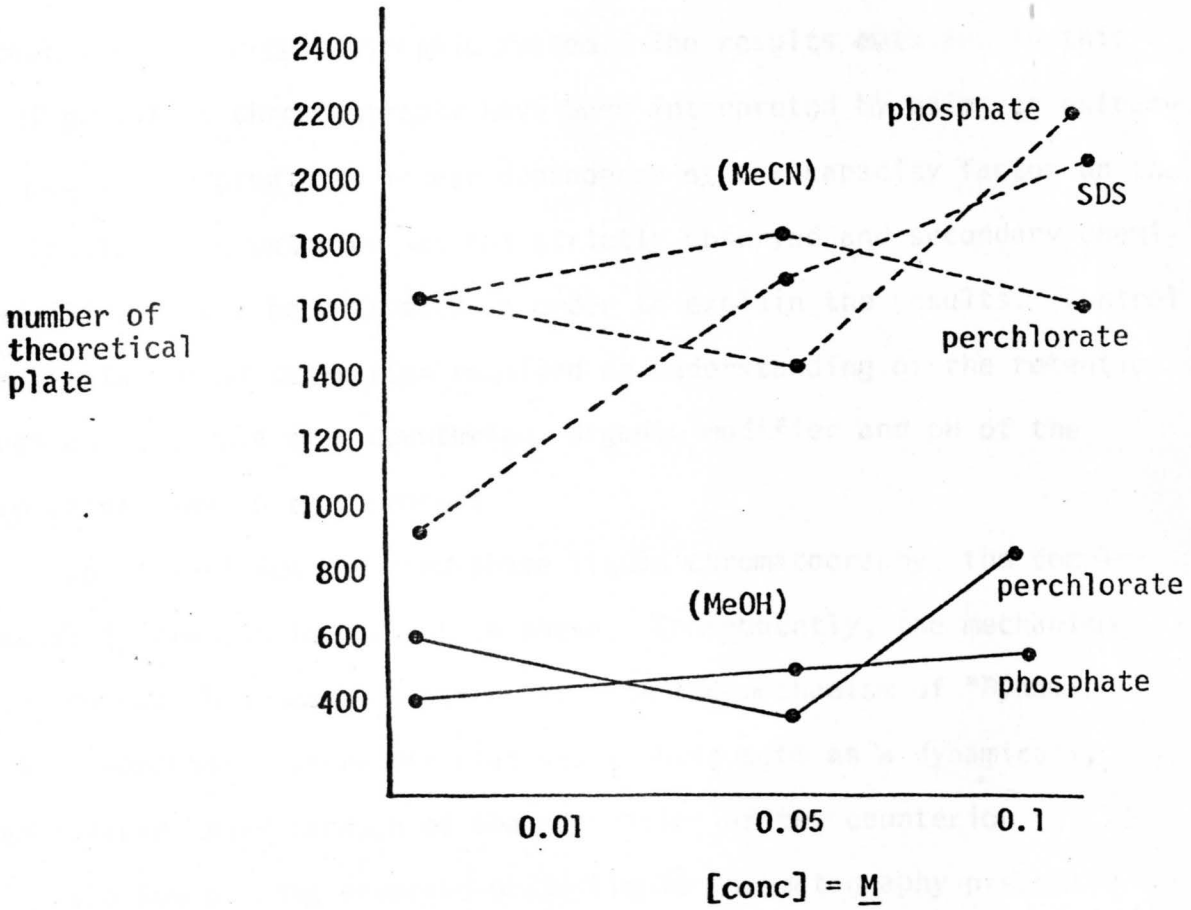
B. —— sulfate-triethylamine system
---- ammonium sulfate system
organic modifier: methanol
pH = 3.5

Table IV Peak symmetry factor, retention time and number of theoretical plates of alkaloids in IP-RPHPLC with different counterion system. Flow rate 1 ml/min, counterion concentration 0.01M, mobile phase water/methanol (50:50), pH 3.5.

Counterion System	Strychnine	Brucine	Nicotine	Paraquat	Atropine
Perchlorate/TEA					
PSF	0.41	0.44	0.66	(0.46)	0.86
Tr	7.8	7.2	4.6	4.6	6
# of T.P.	766	1150	940	177	2130
Sulfate/TEA					
PSF	0.64	0.8	0.56	(0.64)	1
Tr	6	5.4	3.0	4.0	5.0
# of T.P.	1111	900	459	204	625
Phosphate/TEA					
PSF	0.5	0.47	0.55	(0.23)	0.7
Tr	7.6	7.2	4.6	5.4	6.6
# of T.P.	1998	829	880	49	1225
Ammonium Sulfate					
PSF	0.33	0.35	--	0.04	0.35
Tr	9.4	7.6	--	7.6	7.2
# of T.P.	520	499	--	20	576

Fig. 12 Number of theoretical plates as a function of counterion concentration in perchlorate, phosphate and dodecyl sulfate system.

# of T.P. = $16\left(\frac{Tr}{W}\right)^2$



CONCLUSION

The wide use of ion-pairing reversed-phase liquid chromatography arises from the general simplicity of the method, the broad range of substances that can be chromatographed and the inherent selectivity and efficiency of the chromatographic system. The results obtained in this type of partition chromatography have been interpreted by using an extraction model. The predicted linear dependence of the capacity factor on the concentration of counterion was not strictly observed and secondary chemical equilibria have been invoked in order to explain the results. Control and manipulation of separation required an understanding of the retention process and the role that counterion, organic modifier and pH of the mobile phase play in that process.

In ion-pairing reversed-phase liquid chromatography, the complexing agent is present in the mobile phase. Consequently, the mechanism of the separation process is different from the mechanism of "Dynamic liquid ion-exchange",where the stationary phase acts as a dynamically coated ion-exchanger because of the adsorption of the counterion.

The ion-pairing reversed-phase liquid chromatography presented here proved to be simple, rapid, versatile and reproducible. Changing from one system to another system required an equilibrium time of 20 minutes. The easy regulation of the retention of both alkaloids and quaternary ammonium compounds by means of changing pH, organic modifier, the nature of the counterion and its concentration in the mobile phase has made this chromatography system highly suitable for the separation of samples which are closely related and with widely different characters.

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